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Distribution of Catalase, Ribonuclease and Superoxide Dismutase Modified by Monomethoxy(Polyethylene Glycol) into Rat Central Lymph and Lymphatic Nodes

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Summary

The plasma-lymphatic distribution of ribonuclease (RNase), superoxide dismutase (SODase), and catalase (CTase) modified by monomethoxy (polyethylene glycol) (mPEG) was studied in rats. The lymphatic bioavailability (F_L) of individual enzymes administered intravenously was determined on the basis of plasmatic and lymphatic concentration curves. It was concluded that F_L values depend on enzyme-adduct molecular weight (m.w.). The highest F_L value was found in mPEG-RNase (the lowest m.w.), medium value in mPEG-SODase (intermediate m.w.), and the lowest one in mPEG-CTase (the highest m.w.). The binding of these enzymes in the lymphatic tissue of iliac, intestinal, brachial and neck nodes was also proportional to their molecular weight. The lymphatic binding was dependent on the node localization, higher concentrations being found in the iliac and neck nodes in contrast to the other nodes (intestinal, brachial).

Key words

Ribonuclease – Superoxide dismutase – Catalase – mPEG modification – Lymphatic bioavailability

Introduction

The use of enzymes in systemic therapy seems to be an important challenge to pharmaceutical research especially now when, thanks to genetic engineering, many new useful enzymes are being obtained. However, problems such as rapid elimination, difficulties in targeting and immunological reactions limit their use in man. The various solutions proposed concern the entrapment of enzymes into liposomes, erythrocytes or microcapsules, the compartmentalization into extracorporeal shunts or a covalent chemical modification of the enzyme surface by soluble, non-toxic, non-immunogenic polymers (Holcenberg and Roberts 1981, Davis *et al.* 1990). Among the various polymers proposed, monomethoxy(polyethylene glycol) (mPEG) seems to be the most promising from this point of view (Fuertges and Abuchowski 1990, Veronese *et al.* 1992). Usually, the modified enzymes were found to be useful for increasing body residence time (half-time) and reducing antigenicity and immunogenicity. Enzymes modified by mPEG, adenosine deaminase and asparaginase, have already received FDA approval, while many others are in advanced stage of clinical investigation (Nucci *et al.* 1991).

Many reports concerning physico-chemical, pharmacological, toxicological, immunological and therapeutical investigations were reported and the results have been summarized in recent reviews or books (Davis *et al.* 1990, Fuertges *et al.* 1990, Veronese *et al.* 1992, Nucci *et al.* 1991). But investigations regarding the transport of mPEG-enzyme adducts into the extravascular space, especially the lymphatic system, and their binding in the tissue of lymphatic nodes, are still lacking.

As a contribution to a better understanding of the fate of these new therapeutically useful agents, we investigated the distribution of some enzymes between the plasma and lymphatic system, i.e. lymph and lymphatic nodes. We used three mPEG-modified enzymes such as superoxide dismutase (SODase) and catalase (CTase), enzymes with many promising therapeutical applications for their ability to selectively remove the toxic superoxide ion or hydrogen peroxide (Greenwals 1990), and ribonuclease (RNase), an enzyme that is employed in its native state in the treatment of some tumours or encephalitis (Glukhov *et al.* 1976).

Materials and Methods

Materials. CTase from bovine liver, RNase from bovine pancreas and cytidine-2',3'-cyclic monophosphate were purchased from Sigma (St. Louis, MO). Bovine erythrocyte SODase was obtained from Diagnostic Data (Mountain View, CA). Monomethoxy(polyethylene glycol) (mPEG - Mr 5.000) was purchased from Fluka Chemie (Buchs, Switzerland). N-hydroxysuccinimidyl [2',3'-^{3}H] propionate was obtained from the Radiochemical Centre, Amersham (Bucks, UK). All reagents and chemicals were of the finest analytical grade.

Synthesis of monomethoxy(polyethylene glycol)-derivatized enzymes. CTase (20 mg) reacted through its amino groups with activated mPEG, prepared by using ethyl bromacetate-hydroxysuccinimidyl ester as described earlier (Veronese *et al.* 1989). The modification of protein was carried out in 2 ml of 0.2 M borate buffer (pH 8.0) with a molar ratio of mPEG/protein amino groups of 2.5:1 at room temperature for 30 min. The conjugate mPEG-CTase was purified by ultrafiltration (using a PM 10 Amicon membrane), concentrated, and chromatographed by FPLC on a preparative Superose 12 TM column. The solution of 10 mM Na_2HPO_4 and 0.15 M NaCl (pH 7.2) was used as an eluent.

The fractions (2 ml) were examined by absorption at 280 nm and enzymatic activity (Beers and Sizer 1952) for protein elution, by iodine reaction for mPEG elution (Sims and Snape 1980) and for radioactivity if the enzyme was labelled with tritium. The fractions containing the modified enzyme were collected and lyophilized. mPEG-SODase and mPEG-RNase adducts were obtained using the same activated polymer in the same molar ratio as for CTase. The conjugates were purified by ultrafiltration as described above.

The amount of polymer bound to protein was determined from the degree to which the amino groups are modified by measuring the amount of residual amino groups with trinitrobenzenesulfonic acid (Habbeb 1966).

Protein concentrations were determined colorimetrically according to Lowry *et al.* (1951) using the native protein as a reference standard.

Preparation of labelled proteins. mPEG-CTase (15 mg) in 2 ml of 0.1 M borate buffer, pH 8.0, was added to N-hydroxysuccinimidyl [2',3'-^{3}H] propionate (^{3}H-NSP) ($2x10^{-2}$ mCi), an acylating agent, which reacts with free amino groups in the protein molecules to attach the ^{3}H-label group by an amide bond (Yang *et al.* 1982). The reaction mixture was stirred for 15 min at room temperature. The labelled proteins were purified from the excess of radioactive reagent by ultrafiltration using 10 mM CH_3COONH_4, pH 6.5, as an eluent, and from the unreacted polymer by gel filtration chromatography on a Superose 12 column in a FPLC system. mPEG-CTase adduct had a specific activity of 0.45 μCi/mg. The same technique with ^{3}H-NSP described for mPEG-CTase was used for the mPEG-SODase and mPEG-RNase derivatives to obtain a specific radioactivity of 0.40 μCi/mg and 0.97 μCi/mg, respectively.

Animals. The experiments were carried out on Wistar rats weighing 250–280 g. The animals were deprived of food for 18 h but allowed water *ad libitum*.

Cannulation, sampling. The experiments were performed under pentobarbitone general anaesthesia (Pentobarbital inj., Spofa, i.p., 35 mg/kg). Blood samples were withdrawn from one of the cannulated carotid arteries, and lymphatic samples from the thoracic duct cannulated in the neck region (Lamka *et al.* 1986). The blood withdrawal intervals were 2, 7, 15, 30, 45, 60, 90 and 120 min. The samples were centrifuged and plasma was used for the determination of enzymatic concentration. The lymphatic samples were collected at 10-min intervals.

In the 120th min of experiment, the rats were killed and the iliac, intestinal, brachial and neck lymphatic nodes were excised. The nodes were weighed and dissolved in a mixture of 2 M KOH and ethanol (50:50) at 60 °C within 24 hours. The resultant solution was neutralized with H_3PO_4 and the ^{3}H-radioactivity of the sample was determined.

Administration of enzymes. Tritium labelled enzymes were administered intravenously (i.v.) into the saphenous vein as a bolus in doses of 4.36 mg/kg (mPEG-^{3}H-RNase), 6.40 mg/kg (mPEG-^{3}H-CTase), and 5.59 mg/kg (mPEG-^{3}H-SODase).

Determination of enzymes. Radioactivity was determined on a Beta Spectrometer (Tesla) in plasma, lymph and solubilized node samples. The amounts of the enzymes in the biological samples were calculated with reference to standard samples of known activity.

Mathematical evaluation. The Adstat software (TriloByte Inc., Prague 1990) was used for the areas under the concentration curves determinations (AUC_L – area under the lymphatic curve, AUC_P – area under the plasmatic curve) in the 0–120 min interval. The lymphatic bioavailability F_L was calculated as a AUC_L/AUC_P ratio (Lamka *et al.* 1989). The numerical values are shown as means ± standard deviation (SD).

Results

The preparation of the mPEG-modified enzymes was carried out according to a procedure based on the reaction of excess mPEG, activated as an active ester, with the available amino groups of the enzyme. The extent of protein modification, expressed as the percentage of available protein amino groups containing the bound polymer, the approximate molecular weight of the adducts and the residual enzymatic activity are shown in Table 1. The results demonstrate that, using the employed procedure, a high degree of substitution could be obtained without a great loss of enzymatic activity.

Table 1
Extent of polymer modification, molecular weight and residual activity of mPEG-modified enzymes

Enzyme	% of enzyme amino groups with bound mPEG	Molecular weight	Residual enzymatic activity (%)
mPEG-RNase	80	58.000	75
mPEG-SODase	85	120.000	90
mPEG-CTase	50	300.000	80

mPEG-RNase: modified ribonuclease; mPEG-SODase: modified superoxide dismutase; mPEG-CTase: modified catalase. Molecular weight was calculated from the weight of the native enzyme added to the weight of the calculated number of polymer chains bound to the protein. The residual enzymatic activity of RNase, SODase and CTase was evaluated by the method reported elsewhere (Beers and Sizer 1952, Crook et al. 1960, Paoletti et al. 1986).

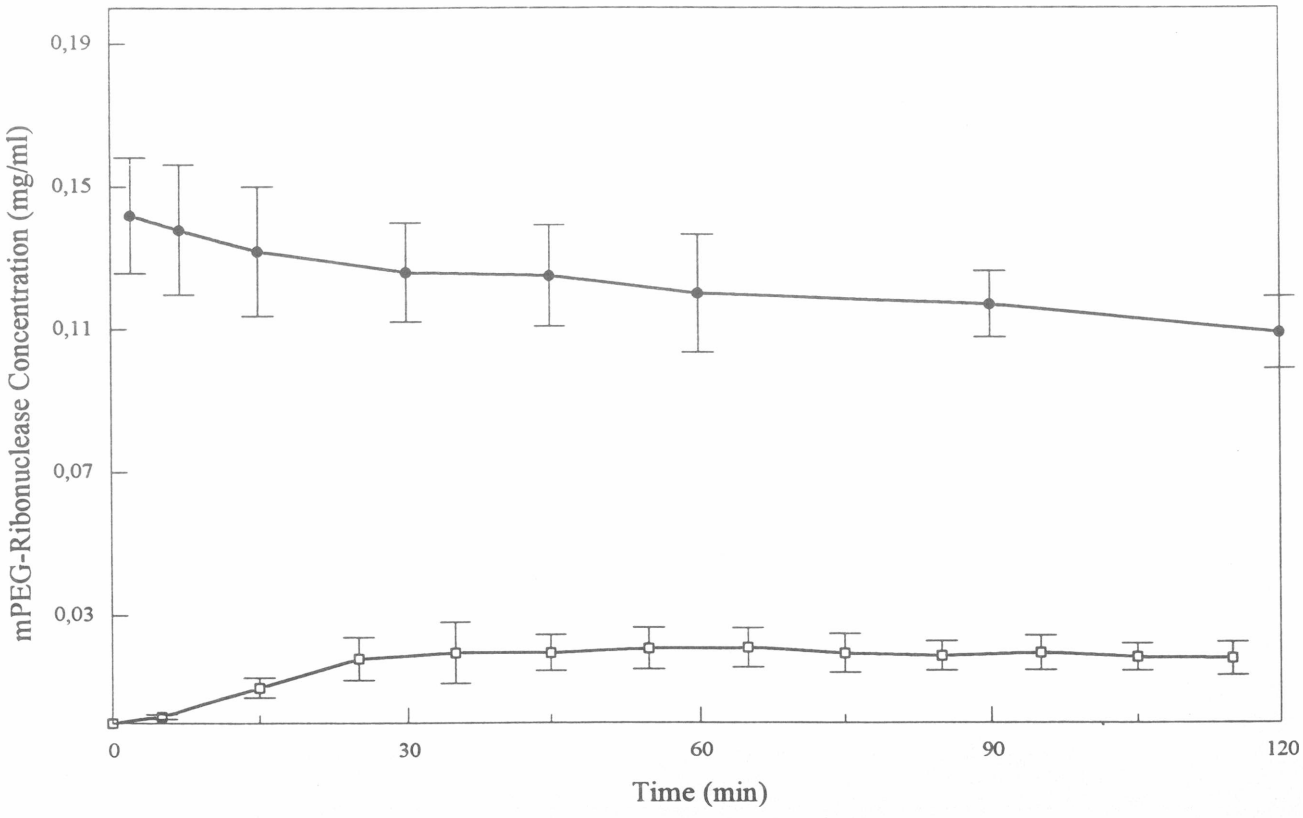

Fig 1
The course of plasmatic (full dots) and lymphatic (open squares) concentrations of mPEG-ribonuclease.

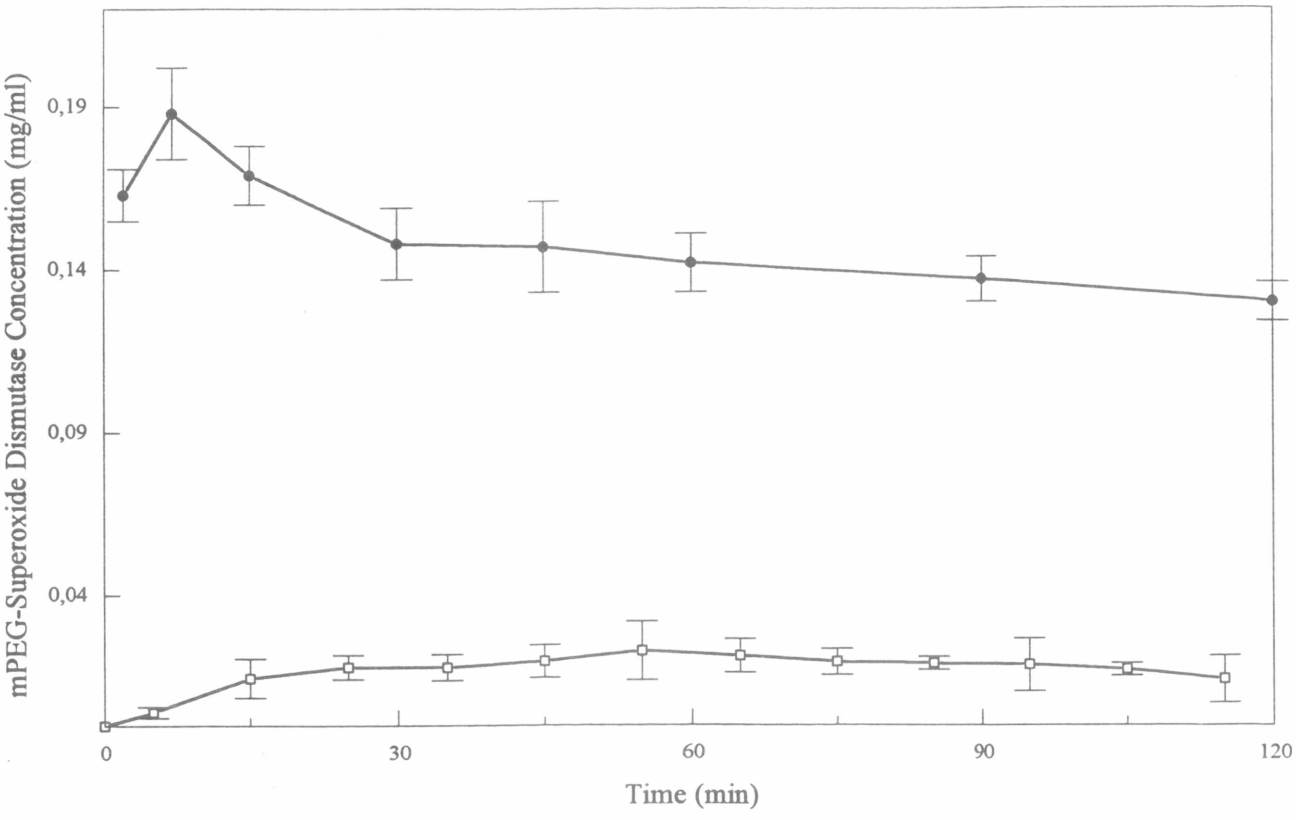

Fig 2
The course of plasmatic (full dots) and lymphatic (open squares) concentrations of mPEG-superoxide dismutase.

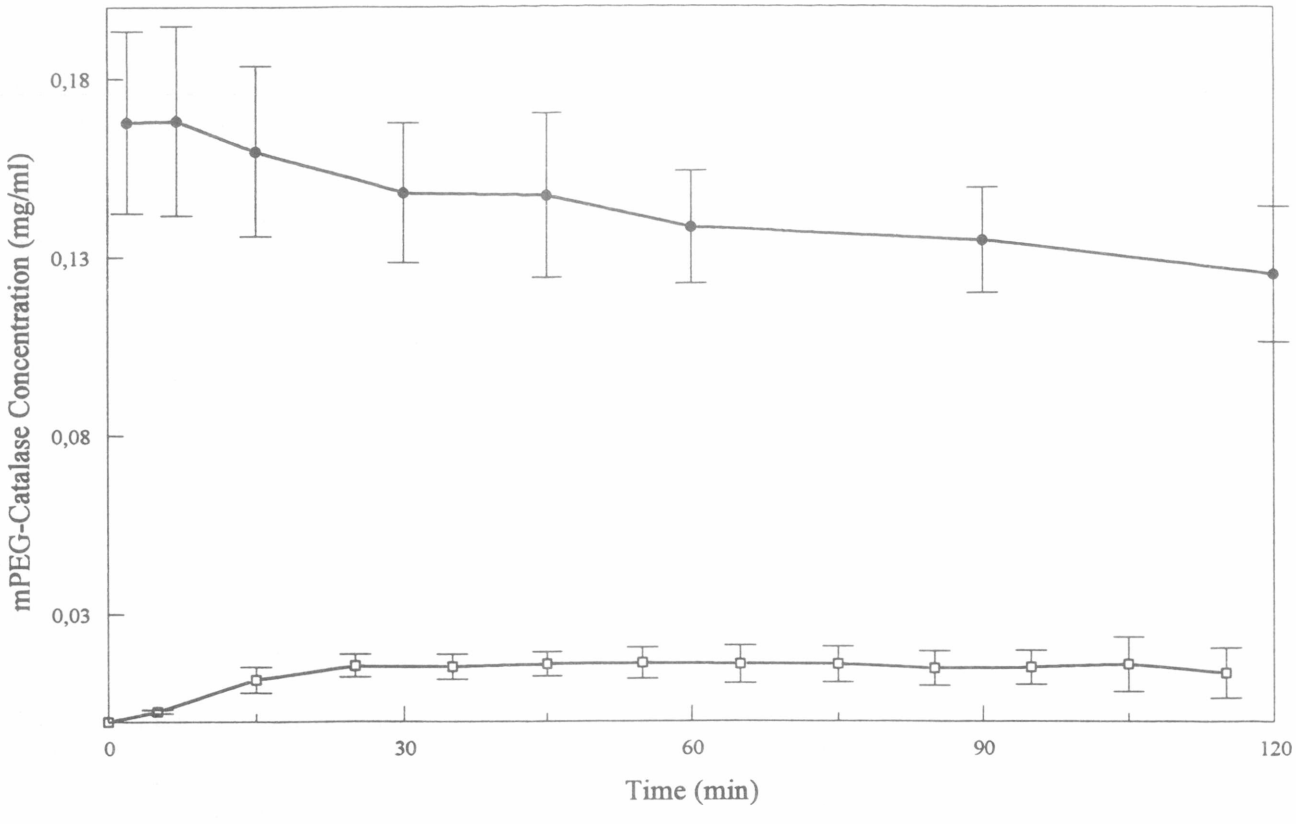

Fig 3
The course of plasmatic (full dots) and lymphatic (open squares) concentrations of mPEG-catalase.

Table 2
Lymphatic bioavailability (F_L) of mPEG modified enzymes

Enzyme	F_L
mPEG-RNase	0.138±0.034
mPEG-SODase	0.114±0.027
mPEG-CTase	0.096±0.010

For further explanations see Table 1.

The concentration profiles of mPEG-RNase, mPEG-SODase and mPEG-CTase in the plasma and lymph are reported in Figs 1–3. In all of the modified enzymes there were large differences in their concentrations. A course of the concentration curves is very similar and is characterized by slow plasmatic decrease and a slow lymphatic increase.

A quantitative comparison of the plasma-lymphatic transport is given in Table 2 as lymphatic bioavailability. The lowest value was obtained for mPEG-CTase, a medium one for mPEG-SODase, and the highest one for mPEG-RNase. To study the distribution of enzymes in the lymphatic system their concentration in lymphatic nodes was evaluated after 120 min of the experiment. The results are reported in Table 3 as the enzyme concentrations in four different groups of lymphatic nodes : iliac, intestinal, brachial and neck. The differences in enzymatic concentrations observed in lymphatic nodes depend upon the enzyme investigated and the localization of the node. In all cases the lowest concentrations were found in the intestinal nodes.

Table 3
Concentrations of modified enzymes in lymphatic nodes (percentage of administered dose/g of lymphatic tissue)

Enzyme	Lymphatic nodes				Mean
	Iliac	Intestinal	Brachial	Neck	
mPEG-RNase	0.350±0.034	0.206±0.050	0.284±0.078	0.412±0.057	0.313±0.055
mPEG-SODase	0.522±0.083	0.316±0.073	0.526±0.246	0.708±0.114	0.518±0.129
mPEG-CTase	0.713±0.093	0.353±0.070	0.510±0.187	0.668±0.230	0.561±0.145
Mean	0.528±0.070	0.292±0.064	0.440±0.170	0.596±0.134	

For further explanations see Table 1.

Discussion

The distribution of both endo- and exogenous substances from the blood bed into the lymph is limited by properties of two barriers: the blood bed-interstitium barrier and the interstitium-lymphatic bed barrier. There are differences in the degree of permeability between these two barriers, the first one being less permeable. Permeability of the blood bed-interstitium barrier is influenced by several factors, the most important of them is the molecular size of the substance. Small substances up to the size of the inulin molecule permeate without restrictions (Parker *et al.* 1984), whereas for larger molecules the permeability also depends upon properties of the barrier (Grotte 1956, Casley-Smith 1982), or on the characteristics of the molecule such as the hydrophilic/hydrophobic ratio, flexibility, charge, etc. (Parker *et al.* 1984). It was assumed that blood, interstitial and lymphatic concentrations of hydrophilic substances of low molecular weight are identical or very similar whereas large differences may occur in higher molecular weight compounds (Casley-Smith 1982).

Rat central lymph, that was considered in the present study, is of mixed origin with a markedly prevalent portion of the lymph from the intestine (Bollman *et al.* 1948, Mann and Higgins 1950). The intestinal plasma-interstitium barrier is of an anatomically combined type (Parker *et al.* 1984). It is characterized as relatively permeable as compared to other endothelious such as skeletal muscles. The permeability of the interstitium-lymphatic bed barrier could be expected to influence the resulting lymphatic concentrations to a lesser extent because it is also very permeable for molecules and particles of substantially larger sizes (for example chylomicrons) than those of the enzymes under study.

The degree of substance distribution into the lymph is indicated by parameter is F_L the value of which represents the ratio of lymphatic AUC/plasmatic AUC. On the basis of F_L values, the extravascular availability of the substances under study can be described more precisely.

The F_L of the mPEG-modified enzymes are very low. It is worth noting that the F_L value for modified RNase is much lower than that for albumin (0.138 as compared to 0.49) although it has a molecular weight lower than albumin (58 000 as compared to 66 000), (Lamka *et al.* 1991). These findings may be explained if one takes into consideration that the hydrodynamic volume of mPEG is higher than the one corresponding to a globular protein of the same molecular weight since the mPEG molecules bound to a protein are described as extended highly hydrated mobile chains (Tsutomu 1985).

There is a correlation between the F_L value and molecular size of the three derivatized proteins investigated here. The F_L decreases from mPEG-RNase to mPEG- SODase and mPEG-CTase, i.e. towards an increasing size. We can therefore conclude that, for this homologous class of compounds, the molecular size is a basic factor in the extravasation process. An additional reason for the reduced extravasation of mPEG-proteins that should also be taken into consideration is their binding to membranes as demonstrated with the endothelium and blood cells (Beckman *et al.* 1988, Caliceti *et al.* 1989). Charge effects, a property that was found of paramount importance in the escaping from blood of proteins derived from other polymers (Fujita *et al.* 1991), will not probably play a role in the present case, since the chemical procedure for mPEG binding used here does not introduce new charges but only reduces the cationic lysine charge where the polymer is bound.

As to the lymphatic node distribution, the results reported in Table 3 indicate that the content of enzymes in intestinal and brachial lymphatic nodes is lower as compared to the iliac or neck nodes. An explanation can be sought in a lower binding ability of the lymphatic tissue of intestinal and brachial nodes but these results can be better explained when taking into consideration the blood supply of all the corresponding tissues under the experimental conditions (general anaesthesia and fasting before the experiment); the blood supply of the intestine and the extremities is lower whereas the neck and pelvis region are better supplied.

The different filtering capacity related to the size of enzymes already demonstrated in the plasma-lymphatic distribution (F_L) also plays a role in the binding of enzymes in the node tissue. This is evident from the data in Table 3 that demonstrate an increasing content of mPEG-modified enzymes in the nodes in relation to the increasing mPEG enzymatic molecular weight. These findings are in good agreement with the properties of some agents (Erkan *et al.* 1985) used in indirect lymphoscinthigraphy.

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